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DISTRIBUTION OF FORSSMAN ANTIGEN IN CHICKENS

NORITOSHI KITAMOTO, KAZUYOSHI IKUTA, HISAKO SHOJI, and SHIRO KATO

Department of Pathology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan

MASAHARU NAIKI

Department of Biochemistry, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan
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SUMMARY The distribution of Forssman antigen in various tissues of chickens was studied by the indirect and direct immunofluorescence (IF) tests using rabbit immune serum against purified Forssman glycosphingolipid (GSL) [GalNAc (α, 1–3)GalNAc(β, 1–3)Gal(α, 1–4)Gal(β, 1–4)Glc-ceramide]. A general finding in all tissue sections was the presence of Forssman antigen in vascular endothelium and perivascular connective tissues. Erythrocytes gave a Forssman positive reaction, but the intensity of fluorescence was slight. Forssman antigen was found in about 20% of all peripheral leukocytes. Fractionation of lymphocytes from peripheral blood into T and B lymphocytes showed that about 30% of the B lymphocytes gave a positive reaction, while only 2–3% of the T lymphocytes gave a positive reaction. About 40% of bone marrow cells expressed Forssman antigen. On the other hand, about 40–50% of adherent cells in in vitro cultures from spleen were Forssman-positive. The antigen was also abundant in hematopoietic organs, such as spleen, thymus and the bursa of Fabricius. Positive reactions in the digestive tract, ovary, testis and skin varied from portion to portion of the tissues. In brain and spinal cord, only the vascular endothelium gave a positive reaction. In peripheral nerves, only the connective tissue stroma around the nerve fibers gave a positive reaction. In other tissues, the distribution of the antigen was generally in accord with the results of Tanaka and Leduc (1956). These positive reactions in the IF test were specifically inhibited by addition of purified Forssman GSL. The distributions of Forssman antigen in tissues were found to be the same in chickens of various ages.

INTRODUCTION

Forssman antigen is known to be present on sheep and goat erythrocytes, and in tissues of guinea pigs, horses, cats, dogs, mice and chickens, but not in those of some other species such as pigs, oxen, rabbits, geese and pigeons. Tanaka and Leduc (1956) demonstrated the

distribution of Forssman antigen in tissues of chickens and other species, such as guinea pigs, cats, dogs, and mice, by the immunofluorescence method using rabbit anti-sheep erythrocyte serum and anti-horse kidney serum as anti-Forssman serum. However, these anti-Forssman sera were not pure and contained other antibodies (Naiki et al., 1971; Ikuta et al., 1981). Later, Forssman antigen was purified from sheep erythrocytes and equine organs as a glycosphingolipid (GSL). It has been identified as a ceramide pentasaccharide with the structure, GalNAc(α, 1–3) GalNAc(β, 1–3)Gal(α, 1–4)Gal(β, 1–4)Glc-ceramide, by Makita et al. (1966), Siddiqui and Hakomori (1971), and Stellner et al. (1973). In this work, using the immunofluorescence (IF) technique with specific rabbit anti-Forssman GSL serum, we examined the distribution of Forssman antigen in normal chicken tissues, including peripheral blood cells, spleen, thymus, the bursa of Fabricius, peripheral nerve, the digestive tract, testis and ovary.

MATERIALS AND METHODS

1. *Preparation of antiserum against Forssman antigen*

Forssman GSL was purified from goat erythrocytes (Naiki et al., 1972). Anti-Forssman serum was prepared by immunizing a rabbit with 1 mg of Forssman GSL mixed with an equal amount of methylated bovine serum albumin as described previously (Kato et al., 1978). The specificity of this serum was described previously (Kato et al., 1978; Ikuta et al., 1981). Briefly, this antiserum at up to high dilution agglutinated sheep erythrocytes, but did not hemolyze bovine erythrocytes, and sheep erythrocyte agglutination antibody was removed by absorption with guinea pig kidney precipitate, but not with bovine erythrocytes. The sheep erythrocyte agglutination of this serum was clearly inhibited by addition of purified Forssman GSL.

2. *Immunofluorescence (IF) tests*

Rabbit anti-Forssman serum, used in the direct IF test, and goat anti-rabbit globulin serum, used in the indirect IF test, were labelled with fluorescein isothiocyanate (FITC). The indirect and direct IF tests were performed as follows. Smear preparations or the tissue sections described below were covered with 30 μl of anti-Forssman serum or FITC-conjugated anti-Forssman serum. After incubation for 30 min at 37°C, the samples were washed three times with 0.01 *M* phosphate buffer (pH 7.0) containing 0.15 *M* NaCl (PBS). Then, for the indirect IF test, samples were covered with 30 μl of FITC-conjugated goat anti-rabbit globulin serum, incubated for 30 min at 37°C, and washed three times with PBS. For the indirect or direct IF tests, samples were then covered with a drop of mounting medium (90% glycerol and 10% PBS) and examined under a fluorescence microscope. The membrane IF test was performed as described previously (Ikuta et al., 1981).

3. *Preparations of tissues and cells from chickens*

One day-old and 1-, 4-, and 25-week-old chickens from a specific pathogen-free flock were used for detection of the Forssman antigen. Chick embryo were obtained from 10- and 13-day-old embryonated eggs. Various organs were promptly removed and frozen. Frozen serial sections of these organs were cut with a cryostat, dried at room temperature and fixed as described below. The Forssman activities in tissue sections were examined by the IF test. One of the serial sections fixed in 10% formalin was stained with hematoxyline-eosin (H-E) for orienting and identifying antigen-positive cells and tissue structures. Peripheral blood or buffy coat cells were smeared on a slide glass, fixed and examined by the IF test. Lymphocytes in peripheral blood were separated with Lymphoprep as described previously (Kitamoto et al., 1979). Lymphocytes from peripheral blood were fractionated into T and B lymphocytes by the nylon wool method as described by Stinson et al. (1978). Spleen cells were incubated in a plastic petri dish for 2 h at 37°C. The nonadherent cells were gently removed from adherent cells and repeatedly washed with PBS. Viable adherent cells were detached with a rubber policeman and reincubated. This procedure was repeated three times. Chick embryo fibroblasts obtained from a 10-day-old embryo were grown to confluence by incubation for 2 days at 37°C and then detached with a rubber policeman. These cell suspensions were washed, smeared on slide glasses and examined as described above. In some cases, parts of the suspensions were examined by

the membrane IF test.

4. *Erythrocytes and lymphoma-derived cell lines*

As Forssman-positive cells, living or fixed guinea pig kidney cells and sheep erythrocytes were used. As Forssman-negative cells, bovine, rabbit and human erythrocytes were used.

As Marek's disease virus (MDV)-transformed cell lines, MDCC-MSB1 (Akiyama and Kato, 1974), derived from MD lymphoma, and MDCC-BP1 (Sekiya et al., 1977), derived from a transplantable MD tumor, were used. As an avian leukosis virus (ALV)-transformed cell line, LSCC-1104B1 (Hihara et al., 1974), which was derived from a lymphoid leukosis (LL) lymphoma, was used. The BP1 and 1104B1 cell lines were kindly supplied by Dr. Sekiya, BIO Pharmaceuticals, Inc., Japan and Dr. Hihara, National Institute of Animal Health, Japan, respectively. It is known that Forssman antigen is present on the surface membrane of BP1 and 1104B1 cells, but not on that of MSB1 cells (Ikuta et al., 1981).

5. *Fixation of smear preparations and tissue sections*

The tissue sections used for H-E were fixed in 10% formalin. The smear preparations and tissue sections used for the IF test were fixed in acetone or 10% formalin for 10 min. In some cases, tissues were examined without fixation. The effect of temperature during acetone fixation was examined at −20°C, 4°C, room temperature (about 20°C), 37°C and 45°C.

6. *Specificity of antibody-antigen reactions*

For confirmation of the specificity of the IF test, the blocking test with Forssman antigen-active GLS was carried out. Purified Forssman GSL (500 μg) was dissolved in 1 ml of PBS containing 0.05% sodium taurodeoxycholic acid by sonication (Naiki and Murcus, 1974). Thirty μl of Forssman GSL was added to 30 μl of anti-Forssman serum for the first incubation in the direct or indirect IF test.

RESULTS

1. *Conditions for fixations and IF tests*

Forssman antigen was observed in the form of granules, droplets and large clumps in the cytoplasm of the cells, but not in the nuclei, of dried, or acetone- or formalin-fixed smear preparations and tissue sections. The dried smear preparations and tissue sections often became detached from the slide glass during the staining procedure. Fine and large granules of specific fluorescence were more distinct and brighter in acetone-fixed smear preparations and tissue sections than in formalin-fixed ones.

The effect of acetone fixation were examined using guinea pig kidney cells. When these cells were fixed at −20°C, 4°C or room temperature, specific fluorescence was demonstrated in their cytoplasm, but when they were fixed at 37°C or 45°C, little fluorescence was seen. These findings were confirmed with chicken lymphoma line cells, smear preparations and tissue sections.

The distribution of Forssman antigen was found to be the same when examined by the indirect and direct IF tests, but the fluorescence was more and clearer in the indirect IF tests. Therefore, the indirect IF test with smear preparations and tissue sections fixed in cold acetone was used in subsequent studies.

2. *Specificity and Forssman properties of the anti-Forssman GSL used*

Acetone-fixed smear preparations of guinea pig kidney cells and sheep erythrocytes, as Forssman antigen-positive cells, and bovine, rabbit and human erythrocytes, as antigen-negative cells, were stained with anti-Forssman GSL serum in the indirect IF test. The living cells were also examined by the membrane IF test (Table 1). Anti-Forssman serum at over 1: 400 dilution stained the membrane of living cells and the cytoplasm of acetone-fixed cells from guinea pig kidney. The antiserum also stained living (Fig. 1A) and fixed (Fig. 1B) sheep erythrocytes, but not bovine, rabbit or human erythrocytes. Chicken erythrocytes were faintly stained at dilutions of up to 1: 10. Previously, we demonstrated Forssman antigen on the surface membrane of BP1 and 1104B1 cells, but not MSB1 cells (Ikuta et al., 1981). Similarly, acetone-fixed BP1 (Fig. 2A) and 1104B1 (Fig. 2B) cells expressed Forssman

TABLE 1. *Specificity of anti-Forssman glycosphingolipid serum against living and acetone-fixed cells*

Cells		Forssman titer[a]
Guinea pig kidney cells	living[b]	>400
	fixed[c]	>400
Sheep erythrocytes	living	100
	fixed	150
Bovine erythrocytes	living	<5
	fixed	<5
Rabbit erythrocytes	living	<5
	fixed	<5
Human erythrocytes	living	<5
	fixed	<5
Chicken erythrocytes	living	10
	fixed	10
MDCC-MSB1	living	<5
	fixed	<5
MDCC-BP1	living	>400
	fixed	>400
LSCC-1104B1	living	200
	fixed	>400

[a] The highest dilution of antiserum giving a positive reaction in more than 50% of the cells. Antiserum dilutions of 1 : 5, 1 : 10, 1 : 50, 1 : 100, 1 : 150, 1 : 200 and 1 : 400 were used in the indirect IF test.

[b] Living cells were examined by the membrane IF test.

[c] Smear preparations were fixed in cold acetone for 10 min, and examined by the IF test.

antigen in their cytoplasm, but MSB1 cells did not (Table 1). In general, acetone-fixed cells were stained at a higher dilution than living cells.

3. *Distribution of Forssman antigen in various cells and tissues from chickens*

The results of observations are summarized in Table 2 and described in detail below. The distribution of Forssman antigen in chick embryos and chickens of various ages were compared. The distribution of Forssman antigen was almost the same in chickens of different

TABLE 2. *Summary of the distribution of Forssman antigen in chickens*

Cells or tissues	Dilution of antiserum[a]		
	10	50	150
Blood cells			
Erythrocytes	>90%[b]	−[c]	−
Granulocytes and lymphocytes[d]	20%	20%	10%
Lymphocyte fraction[e]	30%	20%	NT[f]
T lymphocyte fraction[e]	<3%	<2%	<2%
B lymphocyte fraction[e]	30%	30%	20%
Adherent cells[g]	50%	40%	NT
Chick embryo fibroblasts[h]	80%	60%	40%
Bone marrow cells[d]	40%	40%	30%
Spleen			
Red pulp	###	##	+
White pulp	##	+	+
Trabeculae and Capsule	##	+	+
Vessels	##	##	+
Thymus			
Medulla	##	+	+
Cortex	##	+	+
Septa, Capsule and Vessels	###	##	+
Bursa of Fabricius			
Medulla	###	##	+
Cortex	##	+	+
Capillary network, Capsule and Connective tissue framework	###	##	+
Blood vessels			
Endothelium	###	##	+
Media	+	±	−
Elastic membrane	−	−	−
Adventitia	##	+	±
Cerebrum, Cerebellum and Spinal cord			
Nerve cells, Neuroglia and Nerve fibers	−	−	−
Vessels	##	+	+
Peripheral nerve			
Nerve fibers	−	−	−
Connective tissue stroma	###	##	+
Ovary			
Medulla and Cortex	##	+	+

Continued . . .

TABLE 2. Continued.

Cells or tissues	Dilution of antiserum[a]		
	10	50	150
Tunica albuginea	++	+	+
Theca folliculi	++	+	+
Testis			
Testicular tubules	−	−	−
Connective tissue	+++	++	+
Tunica albuginea	++	+	+
Digestive tract			
Surface epithelium	+ or −[i]	+ or −[i]	−
Lamina propria	+++	++	+
Muscularis mucosa	−	−	−
Submucosa	++	+	±
Glandular epithelium	±	−	−
Lymph nodules	++	+	+
Muscle layer	±[j]	−	−
Serosa	++	++	+
Pancreas			
Endocrine cells	−	−	−
Exocrine cells	−	−	−
Connective tissue	+++	++	+
Adrenal gland			
Medulla	++	+	+
Cortex	−	−	−
Connective tissue	++	+	+
Skin			
Epidermis	++	+	+
Dermis	++	+	+
Liver			
Hepatic cells	−	−	−
Kupffer's cells	++	+	+
Portal canal			
Endothelium of blood vessels	+++	++	+
Epithelium of bile ducts	+	±	−
Connective tissue	+++	++	+
Kidey			
Nephron	−	−	−
Collecting ducts and tubules	++	+	+
Connective tissue	+++	++	±

Continued . . .

TABLE 2. Continued.

Cells or tissues	Dilution of antiserum[a]		
	10	50	150
Trachea			
Epithelium	+++	++	+
Lamina propria	++	+	±
Glandular epithelium	++	+	±
Muscle and Cartilage	−	−	−
Lung			
Alveolar epithelium	++	+	±
Connective tissue	+++	++	+
Vessels			
Endothelium	+++	++	+
Media	+	±	−
Adventitia	+	±	−
Bronchial			
Epithelium	++	+	±
Muscle and Cartilage	−	−	−
Heart			
Cardiac muscle fibers	±[j]	−	−
Connective tissue stroma	++	++	+
Vessels	+++	++	+
Skeletal muscle fibers			
Muscle fibers	±[j]	−	−
Connective tissue stroma	++	++	+
Vessels	+++	++	+

[a] The indirect IF test was performed using anti-Forssman glycosphingolipid serum.
[b] Erythrocytes stained faintly.
[c] +++, intense fluorescence; ++, moderate fluorescence; +, slight fluorescence; ±, very slight fluorescence; −, no fluorescence.
[d] Percentage of positive cells in total leukocytes.
[e] Percentage of positive cells in total fraction.
[f] Not tested.
[g] Cells from spleen cultured in vitro.
[h] Cells from embryos cultured in vitro.
[i] Antigen was present in the esophagus and proventriculus, but not in other organs.
[j] Faint antigen was present in the surface and cytoplasm.

ages. In the indirect IF test, antiserum was used at dilutions of 1: 10, 1: 50 and 1: 150. The photomicrographs in Figs. 1 to 4 are of living cells or smear preparations fixed in cold acetone. Those in Fig. 5 and 7 to 16 are of frozen sections cut at 4 μm and fixed in cold acetone. All photomicrographs are of samples examined by the indirect IF test with anti-Forssman serum at a dilution of 1: 50.

1) Smear preparations and living cells

a) Blood cells: Living and/or fixed erythrocytes were slightly stained at 1: 10 dilution of anti-Forssman serum, but not at 1: 50 dilution. About 20% of samples of living and/or fixed leukocytes from peripheral blood gave a positive reaction (Fig. 3). In general, the positive cells had the same shape as lymphocytes, but some looked like granulocytes or monocytes. In lymphocyte preparations from peripheral blood, about 20% of the living and/or fixed cells were Forssman-positive. After lymphocyte fractionation, about 30% of the B lymphocyte fraction gave a positive reaction, whereas only 2–3% of the T lymphocyte fraction was positive. Moreover, 40–50% of the living and/or fixed adherent cells in in vitro cultures of spleen gave a positive reaction.

b) Bone marrow: About 40% of the living and/or fixed cells were found to express Forssman antigen (Fig. 4). Various kinds of cells were Forssman-positive.

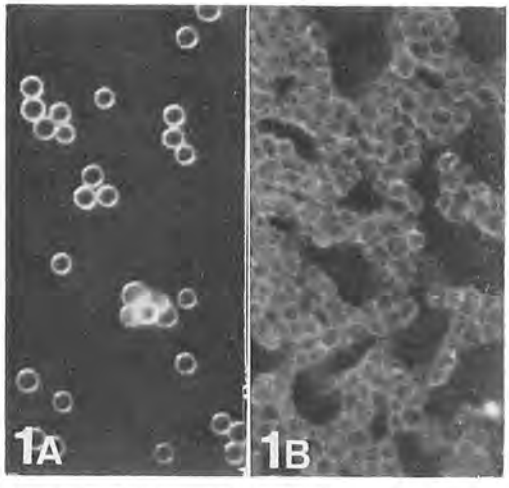

FIGURE 1. Living and fixed sheep erythrocytes. ca. ×600. (A), Living cells examined by the membrane IF test; (B), Smear preparation fixed in cold acetone and examined by the IF test.

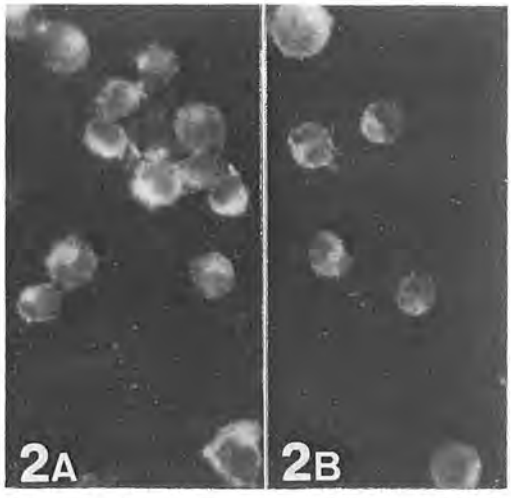

FIGURE 2. Smear preparation of BP1 and 1104B1 line cells fixed in cold acetone. ca. ×600 Forssman antigen was present in the cytoplasm of BP1 (A) and 1104B1 (B) cells.

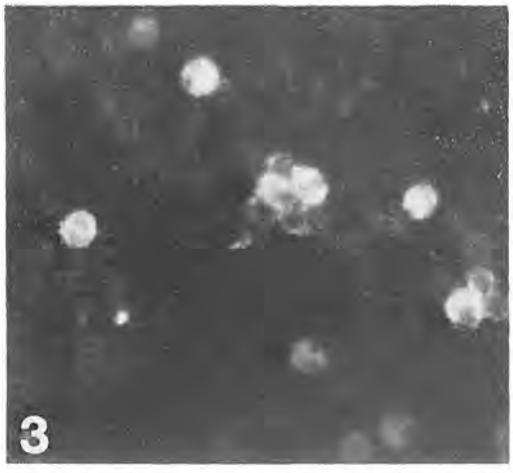

FIGURE 3. A smear preparation of buffy coat cells fixed in cold acetone. ca. ×600. The cytoplasm of several cells shows specific fluorescence.

C) Cultured fibroblasts: Almost all living and/or fixed chick embryo fibroblasts in in vitro cultures from 10-day-old embryos had Forssman antigen on their surface and in their cytoplasm.

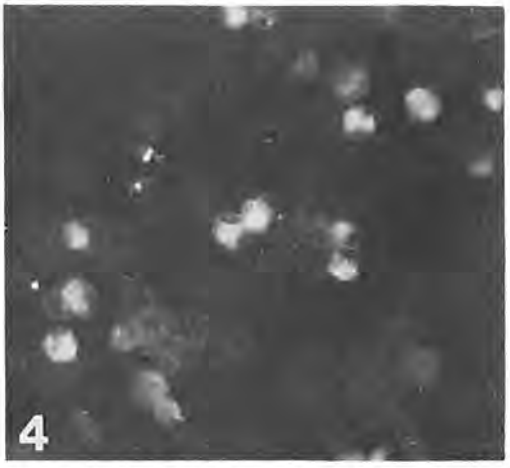

FIGURE 4. A smear preparation of bone marrow cells fixed in cold acetone. ca. ×600. The nuclei, which are sometimes lobed, appear as dark shadow in the fluorescent cytoplasm.

FIGURE 5. A section of spleen fixed in cold acetone. ca. ×200. Specific fluorescence was observed in most cells. Large amounts of antigen were present in cells adjacent to the blood vessels (arrows) and in the red pulp.

2) Tissue sections

In all tissue sections mentioned below, a positive reaction was seen in the vascular endothelium, the connective tissues around blood vessels and so-called interlobular connective tissue, such as trabeculae in the spleen, septa in the thymus and the bursa of Fabricius, the portal canal in the liver, pancreas and testis, and in the capsule of these organs. Therefore, these results are not mentioned again in the descriptions of individual tissue given below.

The specific fluorescence of these cells and tissue sections described below in the IF test appeared bright greenish-yellow and specifically disappeared on additional of Forssman GSL. On the other hand, blue, nonspecific autofluorescence was sometimes observed in certain tissues of chickens, such as the elastic membrane of blood vessels, hyaline cartilage cells in the trachea and lung, and the muscularis mucosa of the digestive tract.

a) Spleen: As shown in Fig. 5, many kinds of cells were stained, but the number of positive cells varied from portion to portion of the tissue. Most cells in the red pulp and in portions closely adjacent to the blood vessels contained Forssman antigen, while some cells in the white pulp showed specific fluorescence.

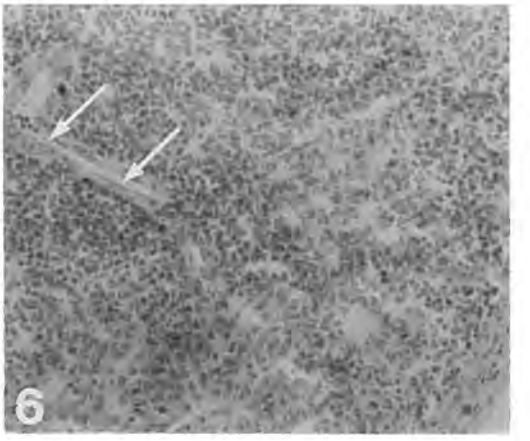

FIGURE 6. Spleen stained with H-E. ca. ×200. One of the serial sections examined in the IF test (shown in Fig. 5) was stained with H-E.

b) Thymus: Fluorescence was observed throughout the tissue, but its intensity was less than that of spleen and the bursa of Fabricius. There was no remarkable difference in the numbers of antigen-positive cells in the medulla and cortex (Fig. 7). Portions of Hassal's corpuscles were Forssman-positive.

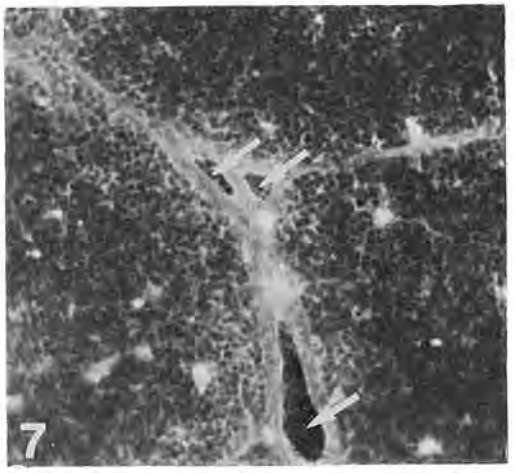

FIGURE 7. Thymus. All sections shown below (Fig. 7–16) were fixed in cold acetone. ca. ×200. Moderate fluorescence was observed. Large amounts of antigen were present in the septa and blood vessel walls (arrows).

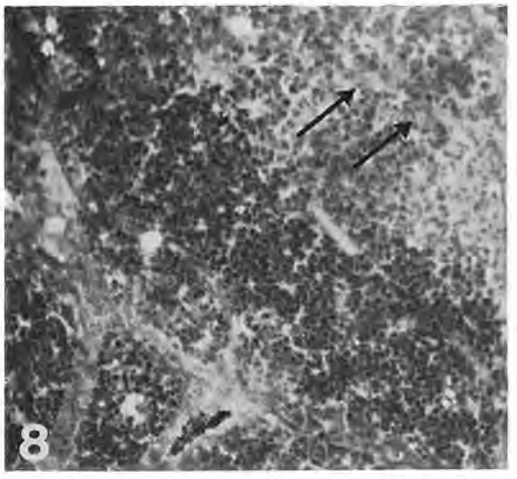

FIGURE 8. Bursa of Fabricius. ca. ×200. Forssman antigen was abundant, especially in the medulla (arrows).

c) Bursa of Fabricius: Forssman antigen was abundant on most cells in sections of the bursa. The cells in the medulla showed stronger fluorescence than those in the cortex (Fig. 8).

d) Blood vessels: In sections of arteries (Fig. 9), the antigen was observed in the endothelium of the intima, and connective tissue cells of the media and adventitia, but the elastic membrane and smooth muscular cells in the media did not show any fluorescence. The external elastic membrane showed intense autofluorescence. In the smaller arterioles, the antigen was limited to the endothelium of the intima. In veins, the antigen was observed in the endothelium and adventitia. The media contained autofluorescence. Endothelial cells of the smaller vessels in all sections described above and below generally showed intense specific fluorescence.

e) Cerebrum, Cerebellum and Spinal cord: The antigen was found only in the endothelium of the vessels: other tissues were devoid of antigen.

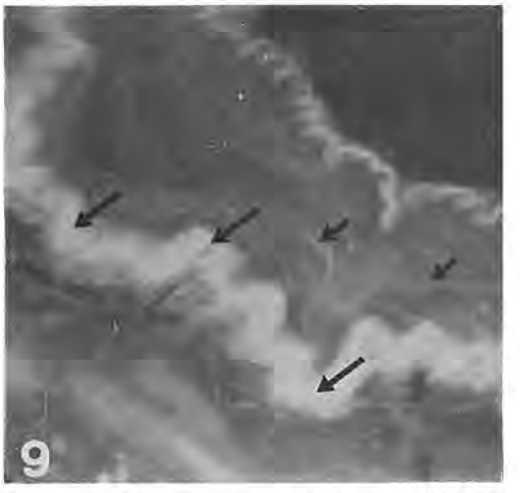

FIGURE 9. A medium-sized artery. ca. ×400. The antigen was present in the endothelium of the intima. In the media, small amounts of Forssman specific fluorescence (short arrows) and autofluorescence were observed. The external elastic membrane contained intense autofluorescence (long arrows).

f) Peripheral nerve: A sample was taken from the sciatic nerve. The nerve fibers themselves contained no antigen, but the connective tissue stroma, so-called endoneurium, perineurium and epinerium around nerve fibers were filled with specific fluorescence (Fig. 10A and 10B).

g) Ovary: An ovary was obtained from a 4-week-old chicken. The antigen was abundant in cells of the cortex, medulla, tunica albuginea and theca folliculi.

h) Testis: A mature testis from a chicken at 25 weeks old was examined. Seminiferous tubules containing sperma did not have the antigen, but the connective tissues between them and the tunica albuginea contained antigen.

i) Digestive tract: The antigen was distributed throughout the digestive tract, for example, in the esophagus, proventiculus, gizzard, small intestine, large intestine and cecum. The antigen was present in the lamina propria and submucosa throughout these regions. However, the epithelium of the mucosal membrane and glandular epithelium in all regions except the esophagus and proventriculus were Forssman-positive. A part from connective tissue, the muscularis mucosa and smooth muscle fibers were Forssman-negative (Fig. 11). Autofluorescence was also present in the muscularis mucosa. Pink and faint blue autofluorescence was seen in the keratinized layer of the gizzard and in the muscle layer, respectively. Lymph nodules in the lamina propria of the cecum contained antigen (Fig. 12).

j) Pancreas: Neither exocrine nor endocrine cells had Forssman antigen.

k) Adrenal gland: The antigen was located in the medullary cells. Except for connective tissues, other cells had no antigen.

l) Skin: Non-specific pink and blue staining was found in the stratum corneum of the epidermis, but specific fluorescence was present in the stratum granulosum. The antigen was also found throughout the dermis, especially in contact with blood vessels and feather follicles.

m) Other tissues: The tissues described below were examined by Tanaka and Leduc

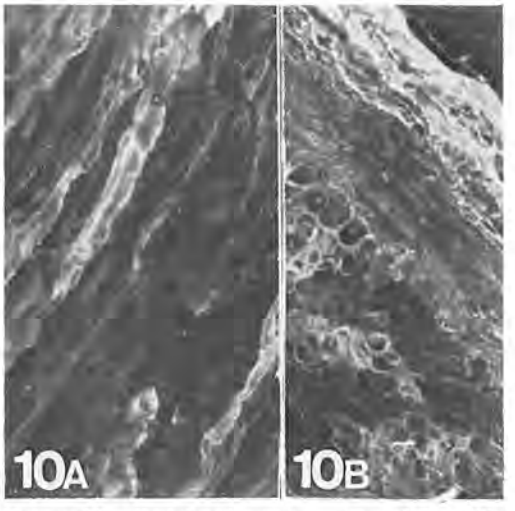

FIGURE 10. Peripheral nerve. ca. ×200. The antigen was not detected in the nerve fibers, but was present in the connective tissue stroma. (A), longitudial section; (B), transverse section.

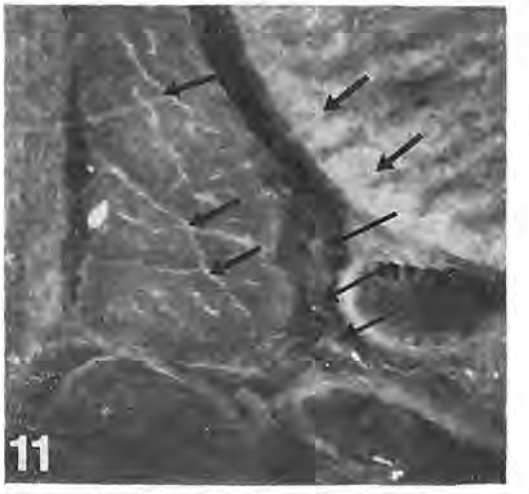

FIGURE 11. Proventriculus. ca. ×100. Forssman antigen was present in the proventricular glands and connective tissue stroma around the smooth muscle fibers (short arrows). Autofluorescence was seen in portions containing stands of the inner layer of the muscularis mucosa (long arrows).

(1956) with FITC-conjugated rabbit anti-sheep erythrocyte serum by the direct IF test. Our results confirmed their findings, although the intensity of our stain in several tissues seemed to be stronger. Briefly, in liver sections, hepatic cells did not show any fluorescence, but the endothelial and Kuppfer's cells were Forssman-positive (Fig. 13). In the kidney, Forssman antigen was present in cells consisting of the smaller collecting tubules (Fig. 14A) and large collecting ducts (Fig. 14B), but not in those of the so-called nephron, that is, the glomerulae and tubules. In the trachea, the antigen was detected in the columnar cells of the epithelium. The glandular epithelium and portions of the lamina propria

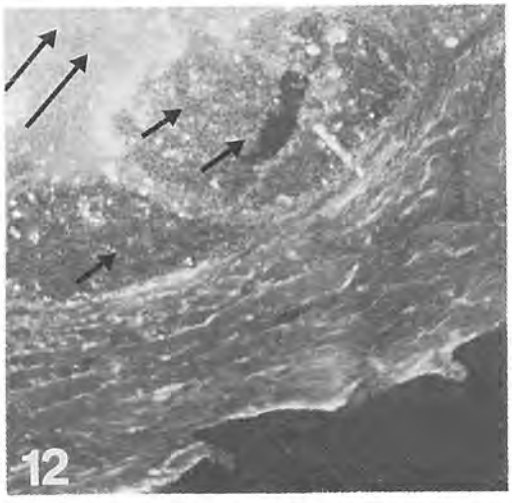

FIGURE 12. Cecum. ca. ×100. Moderate amounts of antigen were present in the lymph nodules (short arrows) and the lamina propria (long arrows).

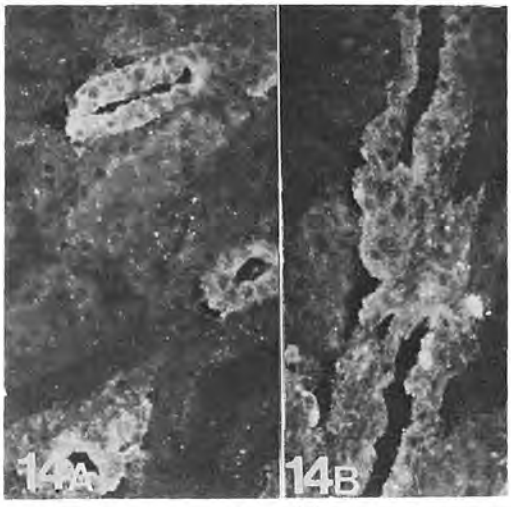

FIGURE 14. Kidney. ca. ×200. The antigen was present in cells which seemed to be collecting tubules (A and B).

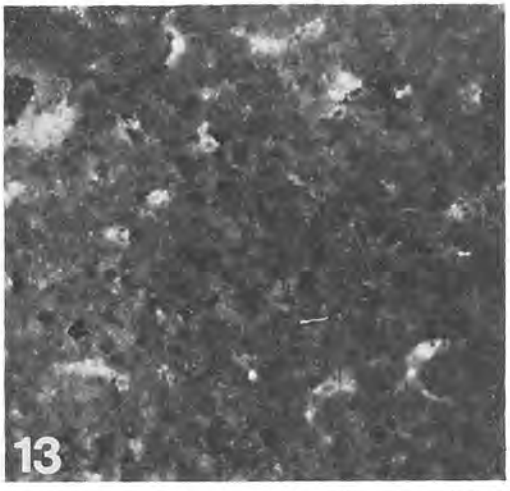

FIGURE 13. Liver. ca. ×200. Hepatic cells had no antigen, but the endothelial cells in sinusoids were Forssman-positive.

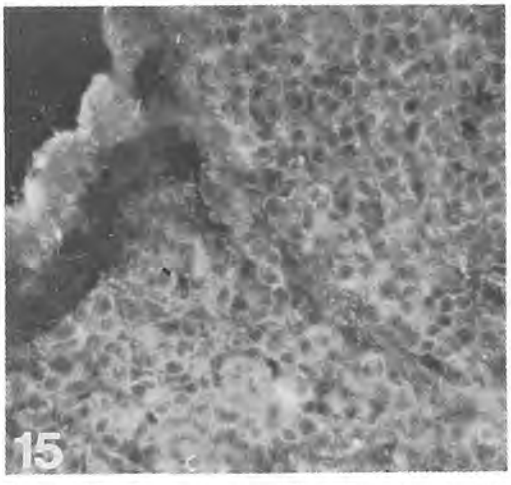

FIGURE 15. Lung. ca. ×400. The alveolar epithelium and the bronchiolar epithelium contained antigen.

contained a little antigen. The cytoplasm of hyaline cartilage cells and the matrix showed blue-white autofluorescence. Similarly, cartilage cells and matrix, and smooth muscle fibers around the blood vessels in the lung were Forssman-negative. However, the alveolar epithelium contained much antigen (Fig. 15). The antigen was also abundant in the epithelium of the bronchioles. The cardiac muscle fibers and skeletal muscle fibers themselves were devoid of intracellular antigen, but the capillary endothelium, the connective tissue cells in the interstices between muscle fibers and the surface of the muscle fibers were Forssman-positive (Fig. 16). The cytoplasm of muscle fibers exhibited faint blue-white autofluorescence.

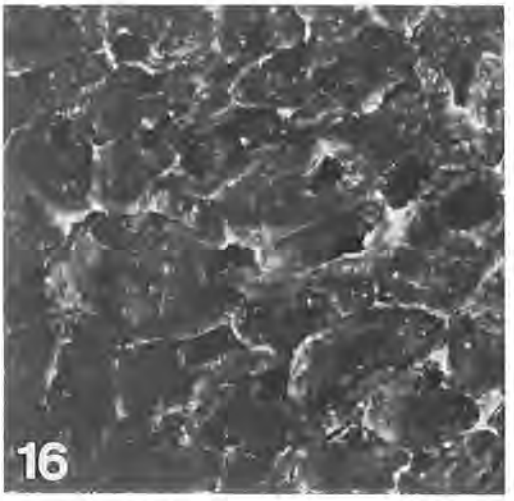

FIGURE 16. Skeletal muscle. ca. ×200. No antigen could be detected in the cytoplasm of the muscle fibers, but it was present on their surface or in the connective tissue stroma. Slight autofluorescence was seen in the cytoplasm of the muscle fibers.

DISCUSSION

In this work, we examined the distribution of Forssman antigen in the tissues of chickens by the IF test using rabbit antiserum against purified GSL. This antiserum consisted mainly of IgG antibodies (4×10^4 $C'H_{50}$ unit/ml in micro-complement fixation) with a small amount of IgM antibodies (1.7×10^4 unit/ml), but both the antibody fractions were highly specific for Forssman GSL and did not cross-react with other GSL antigens of similar structure, such as globoside (blood group P antigen), GalNAc(β, 1–3)Gal(α, 1–4)Gal(β, 1–4)Glc-ceramide or ceramide trihexoside (blood group p^k antigen), Gal(α, 1–4)Gal(β, 1–4)Glc-ceramide (Kato et al., 1978). Methods for purification of specific antibody to GSL antigens have been reported by several workers (Laine et al., 1974; Marcus, 1976; Young et al., 1979). But in the case of Forssman antibody, purification was not necessary, because the antiserum contained a very high titer of specific Forssman antibodies, and thus non-specific antibodies which are naturally present in the serum did not disturb indentification of the specific fluorescence.

In this communication, cells and cryostat sections were fixed in cold acetone. This fixation seems suitable for glycolipid antigens, because GSLs such as Forssman antigen-active substance are not extracted by cold acetone, whereas almost all the membrane cholesterol is removed. Thus the antiserum can be more easily mounted on the tissue section without being repelled by tissue lipid, and antibodies can penetrate into the cells. Usually, finer and brighter specific fluorescence was obtained in acetone-fixed cells and tissue sections than in formalin-fixed ones. Marcus and Janis (1970) fixed tissue sections in 10% formalin or 95% ethanol to detect globoside by the IF technique. But ethanol fixation is not so suitable for glycolipid antigens, because ethanol can dissolve GSLs at room temperature. Baecque et al. (1976) compared ethanol, formalin and acetone fixations for examining the localization of ganglioside GM_1 in frozen cerebellar sections, obtaining the best results with formalin-fixation, but they used acetone at room temperature. If they had used cold acetone, they would have obtained better results, because GSLs are known to be soluble in hot acetone but insoluble in cold acetone.

Forssman antigen was expressed in the cytoplasm of fixed cells as well as on the surface of living cells. Forssman GSL may be associated with membrane components of the plasma membrane and/or intracellular organellae. Chemical analyses of GSLs in subcellular fractions suggested that GSLs might be confined to the plasma membrane and not present intracellular organellae, such as mitchondria, nuclei or the endoplasmic reticulum (Dod and Gray, 1968; Rouser et al., 1968). But IF studies showed that globoside located in the cytoplasm may be present in mitochondria of renal tubular cells (Marcus and Janis, 1970). Our results also suggested that Forssman GSL is present in the cytoplasm of various tissue cells, but its precise intracellular localization requires study by electron microscopy.

A common finding on the tissue localization of Forssman antigen was that it was present in the vascular endothelium and perivascular connective tissues in all sections. Our data are in general agreement with those of Tanaka and Leduc (1956). In addition we tested for Forssman antigen in some other tissues which they did not examine, such as peripheral blood cells, thymus, the bursa of Fabricius, the digestive tract, peripheral nerve, and the ovary. Our finding that Forssman antigen is present in these organs is interesting, because these tissues are susceptible to MD or LL lymphoma in chickens as discussed below. We also examined tissues from chickens of various ages, but found no remarkable change in the distribution of Forssman antigen with age.

A characteristic feature of the distribution of Forssman antigen seems to be its association with the reticuloendothelial system of the vascular system, for example, the reticulum cells or histiocytes in hematopoietic organs, Kuppfer's cells in the liver and the endothelium and adventitial cells of blood vessels. Forssman antigen also seems to be present in the reticular fibers constituting reticular endothelial tissues or fibrous connective tissues. In this connection, it is noteworthy that we found that about 50% of adherent cells from spleen were Forssman-positive and that almost all chick embryo fibroblasts in tissue culture contained Forssman antigen. It was uncertain whether Forssman antigen was present in chicken erythrocytes (Boyd, 1966). We found that they were stained slightly with 1 : 10 dilution of anti-Forssman serum in the indirect IF test. The antigen in chicken erythrocytes was quantitatively less than that in erythrocytes of sheep and goat. About 20% of the lymphocytes and granulocytes in the peripheral blood were Forssman-positive, although the percentage of positive cells differed in different chickens. Fractionation of peripheral lymphocytes into T and B lymphocytes showed that about 30% of the B cells gave a positive reaction, whereas only 2–3% of the T cells were positive. The indirect IF test using anti-T and B cell specific sera on the fractionated T cells and B cells showed that the two fractions were almost pure, but that a few percent of T cells contaminated in the B cell fraction and vice versa. So, the positive reactions in the T cell fraction may be due to contaminating B cells. This result is consistent with our previous findings on splenic T or B lymphocytes (Ikuta et al., 1981). Narasimhan and Murray (1978) extracted GSLs from chicken thymus and the bursa of Fabricius and detected Forssman GSL in both organs, although the content of GSL in the thymus was less than one fifth of that in the bursa. We also detected the antigen in the thymus, but the fluorescence in the thymus was not so bright as in the bursa. These result suggest that mature T lymphocytes may lose Forssman GSL and may be released from the thymus into the blood or lymph.

We found by the indirect membrane IF test with rabbit anti-Forssman GSL serum that Forssman antigen was expressed on avian leukosis virus (ALV)-transformed cell lines and transplantable MD tumor cell lines, but not on MD lymphoma-derived cell lines (Ikuta et al., 1981). ALV-transformed cell lines have been shown to carry a B cell marker, whereas

MD lymphoma-derived cell lines carry a T cell marker (Nazerian and Sharma. 1975; Matsuda et al., 1976; Calnek et al., 1978). This fact is of interest in relation to our finding that the percentage of Forssman-positive cells was high in the B cell fraction, but not in the T cell fraction. The retention or disappearance of Forssman antigen could be a useful marker for differential diagnosis of LL from MD in field work.

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